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Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference	ACC/JR/P32329
2. Patent application number *(The Patent Office will fill in his part)*	9914085.7 16 JUN 1999
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	SmithKline Beecham plc New Horizons Court, Brentford, Middx TW8 9EP, Great Britain
Patents ADP number *(if you know it)*	
If the applicant is a corporate body, give the country/state of its incorporation	58009740003
4. Title of the invention	New Use
5. Name of your agent *(if you have one)*	CORPORATE INTELLECTUAL PROPERTY
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	SMITHKLINE BEECHAM PLC TWO NEW HORIZONS COURT BRENTFORD MIDDLESEX TW8 9EP
Patents ADP number *(if you know it)*	58009740004
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number	Country — Priority application number *(if you know it)* — Date of filing *(day / month / year)*
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application — Date of filing *(day / month / year)*
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer yes if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is named as an applicant, or c) any named applicant is a corporate body. See note (d))*	

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	1
Description	9
Claim(s)	3
Abstract	1
Drawings	2

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10. If you are also filing any of the following, state how many against each item.

Priority Documents

Translations of priority documents

Statement of inventorship and right to grant of a patent *(Patents Form 1/77)*

Request for preliminary examination and search *(Patents Form 9/77)*

Request for substantive examination *(Patents Form 10/77)*

Any other documents *(please specify)*

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11. We request the grant of a patent on the basis of this application

Signature A C Connell   Date 16-Jun-99

A C Connell

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12. Name and daytime telephone number of person to contact in the United Kingdom   A C Connell 01279 644395

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CONTINUATION SHEET

Reference: ACC/JR/P32329

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New Use

This invention relates to new uses for carbohydrate-based surfactant compounds. Such uses include facilitating the transfer of compounds into cells for drug delivery and facilitating the transfer of oligonucleotides and polynucleotides into cells for gene expression studies or gene therapy. The invention also relates to new carbohydrate-based surfactant compounds and methods for their production.

Surfactants are substances that markedly affect the surface properties of a liquid, even at low concentrations. For example surfactants will significantly reduce surface tension when dissolved in water or aqueous solutions and will reduce interfacial tension between two liquids or a liquid and a solid. This property of surfactant molecules has been widely exploited in industry, particularly in the detergent and oil industries. In the 1970s a new class of surfactant molecule was reported, characterised by two hydrophobic chains with polar heads which are linked by a hydrophobic bridge (Deinega,Y *et al.*, *Kolloidn. Zh.* **36**, 649, 1974). These molecules, which have been termed "gemini" (Menger, FM and Littau,CA, *J.Am.Chem.Soc.* **113**, 1451, 1991), have very desirable properties over their monomeric equivalents. For example they are highly effective in reducing interfacial tension between oil and water based liquids and have a very low critical micelle concentration. Recently, Pestman *et al* have reported the synthesis and characterisation of nonionic carbohydrate-based gemini surfactants (Pestman, JM et al, Langmuir, **13**, 6857-6860, 1997).

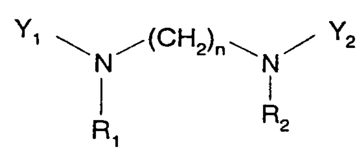
Cationic surfactants have been used *inter alia* for the transfection of polynucleotides into cells in culture, and there are examples of such agents available commercially to scientists involved in genetic technologies (for example the reagent Tfx^{TM}-50 for the transfection of eukaryotic cells available from Promega Corp. WI, USA).

The efficient delivery of DNA to cells *in vivo*, either for gene therapy or for antisense therapy, has been a major goal for some years. Much attention has concentrated on the use of viruses as delivery vehicles, for example adenoviruses for epithelial cells in the respiratory tract with a view to corrective gene therapy for cystic fibrosis (CF). However, despite some evidence of successful gene transfer in CF patients, the adenovirus

route remains problematic due to inflammatory side-effects and limited transient expression of the transferred gene. Several alternative methods for *in vivo* gene delivery have been investigated, including studies using cationic surfactants. Gao,X *et al.* (1995) *Gene Ther.* **2**, 710-722 demonstrated the feasibility of this approach with a normal human gene for CF transmembrane conductance regulator (CFTR) into the respiratory epithelium of CF mice using amine carrying cationic lipids. This group followed up with a liposomal CF gene therapy trial which, although only partially successful, demonstrated the potential for this approach in humans (Caplen, NJ. *et al., Nature Medicine*, 1, 39-46, 1995). More recently other groups have investigated the potential of other cationic lipids for gene delivery, for example cholesterol derivatives (Oudrhiri,N *et al. Proc.Natl.Acad.Sci.* **94**, 1651-1656, 1997). This limited study demonstrated the ability of these cholesterol based compounds to facilitate the transfer of genes into epithelial cells both *in vitro* and *in vivo*, thereby lending support to the validity of this general approach.

These studies, and others, show that in this new field of research there is a continuing need to develop novel low-toxicity surfactant molecules to facilitate the effective transfer of polynucleotides into cells both *in vitro* for transfection in cell-based experimentation and *in vivo* for gene therapy and antisense treatments. The present invention seeks to overcome the difficulties exhibited by existing compounds.

The invention relates to the use of carbohydrate-based surfactant compounds having the general formula (I):



(I)

wherein Y_1 and Y_2, which may be the same or different, are carbohydrate groups, preferably sugars;

R_1 and R_2, which may be the same or different, are selected from:

a) hydrogen;

b) $C_{(1-24)}$ alkyl group; or

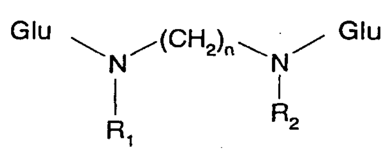
c) $C_{(1-24)}$ alkyl carboxy group;

and n is from 1 to 10;
or a salt, preferably a pharmaceutically acceptable salt thereof,
for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*.

Preferably the compound is symmetrical, that is the groups R_1 and R_2 are the same, and Y_1 and Y_2 are the same. The molecular symmetry allows these compounds to be referred to as "gemini" surfactants.

In a preferred embodiment, the carbohydrate groups Y_1 and Y_2 are sugars, attached to the nitrogen via a reduced imine bond. Such sugars include monosaccharides such as glucose and fructose, disaccharides such as lactose and more complex sugars, for instance sugars based on cellulose.

In a particularly preferred embodiment, Y_1 and Y_2 are glucose; the compounds having the general structure of formula (II):



(II)

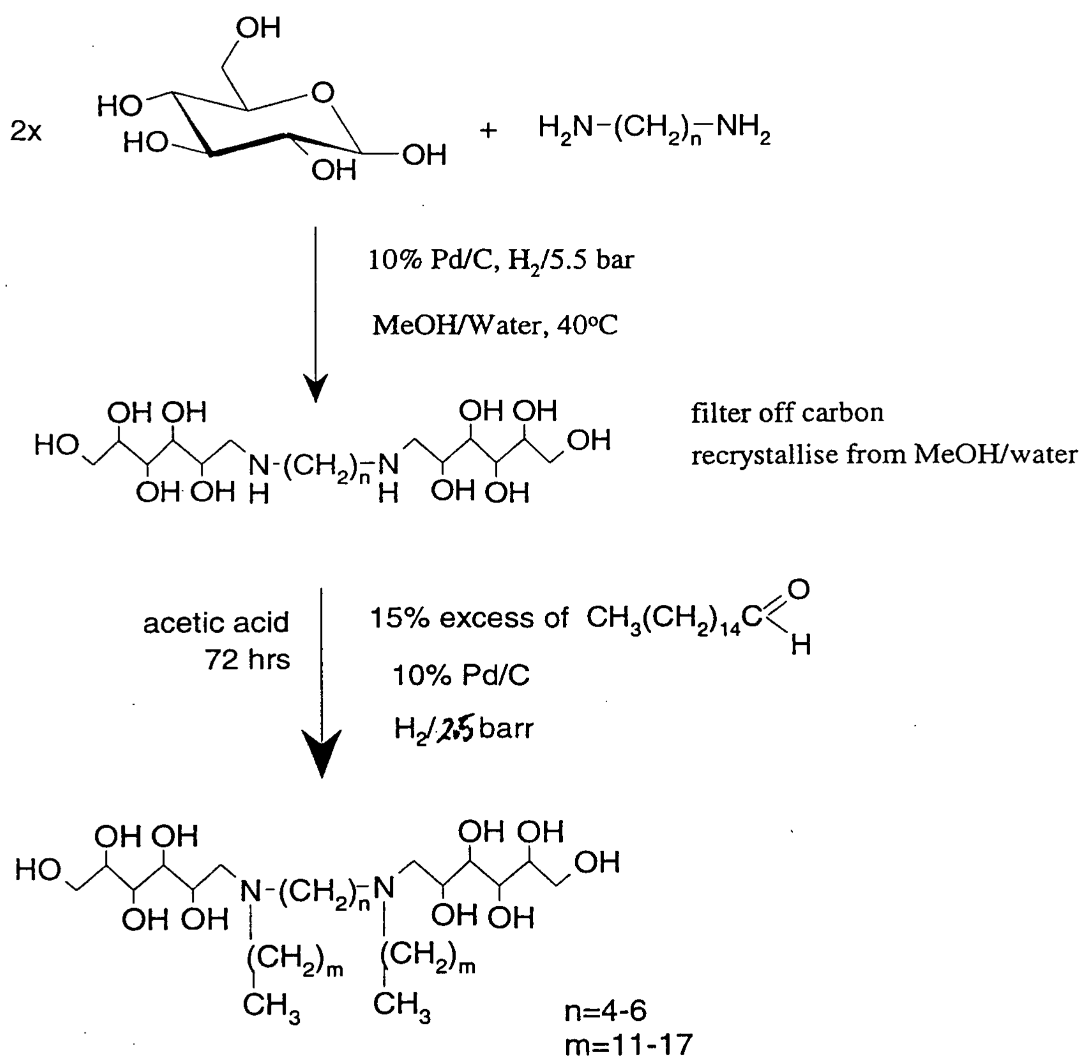
wherein Glu is glucose in open chain form (glucitol) linked via the C-1 (aldehyde carbon), and R_1, R_2 and n are as hereinbefore defined.

In a further preferred embodiment R_1 and R_2 are alkyl groups of chain-length $C_{(10\text{-}20)}$, most preferably $C_{(12\text{-}18)}$, and n is between 2 and 8, most preferably 4 or 6. Such compounds are new and form part of the present invention.

The present invention shows the surprising finding that carbohydrate-based surfactants are highly efficient agents for facilitating the transfection of polynucleotides into cells.

Compounds of formula (I) in which R_1 and R_2 are not both $C_{(1\text{-}24)}$ alkyl carboxyl groups are new. Accordingly, in a further aspect, the present invention provides for compounds of formula (I) in which one of R_1 or R_2 is an alkyl group of chain-length $C_{(1\text{-}24)}$, and the other is a $C_{(1\text{-}24)}$ alkyl carboxy group.

Compounds of the present invention may be prepared from readily available starting materials using synthetic chemistry well known to the skilled person. A general process for preparing carbohydrate-based surfactant compounds comprises the addition of carbohydrate groups at the amine ends of an alkyl diamine compound. The following is a general scheme for the synthesis of the sugar-based compounds of the invention, as illustrated for glucose-based compounds:



filter off carbon
evaporate solvent
deprotonate using ion exchange in EtOH/MeOH
recrystallise from EtOH, MeOH
or acetonitrile

For $R_1 = C_{(1-24)}$ alkyl carboxy, the second step will be the formation of an amide bond, using a suitable acylating agent, for instance an activated derivative of the corresponding acid.

The processeses described above are for the synthesis of symmetrical, that is "gemini", carbohydrate-based surfactants. Non-symmetrical carbohydrate-based surfactants of the invention can be prepared by introducing asymmetry, for example at the primary amines of the diamine, by using different protecting groups.

In a preferred embodiment, the carbohydrate-based surfactant compounds are used to facilitate the transfer of oligonucleotides and polynucleotides into cells to achieve an antisense knock-out effect, for gene therapy and genetic immunisation (for the generation of antibodies) in whole organisms. In a further preferred embodiment, the carbohydrate-based surfactant compounds are used to facilitate the transfection of polynucleotides into cells in culture when such transfer is required, in, for example, gene expression studies and antisense control experiments among others. The polynucleotides can be mixed with the compounds, added to the cells and incubated to allow polynucleotide uptake. After further incubation the cells can be assayed for the phenotypic trait afforded by the transfected DNA, or the levels of mRNA expressed from said DNA can be determined by Northern blotting or by using PCR-based quantitation methods for example the Taqman® method (Perkin Elmer, Connecticut, USA). Compounds of the invention offer a significant improvement, typically between 3 and 6 fold, in the efficiency of cellular uptake of DNA in cells in culture, compared with compounds in the previous art. In the transfection protocol, the gemini compound may be used in combination with one or more supplements to increase the efficiency of transfection. Such supplements may be selected from, for example:

(i) a neutral carrier, for example dioleyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289);

(ii) a complexing reagent, for example the commercially available PLUS reagent (Life Technologies Inc. Maryland, USA) or peptides, such as polylysine or polyornithine peptides or peptides comprising primarily, but not exclusively, basic amino acids such as lysine, ornithine and/or arginine. The list above is not intended to be exhaustive and

other supplements that increase the efficiency of transfection are taken to fall within the scope of the invention.

In still another aspect, the invention relates to the transfer of genetic material in gene therapy using the compounds of the invention.

Yet another aspect of the invention relates to methods to effect the delivery of non-nucleotide based drug compounds into cells *in vitro* and *in vivo* using the compounds of the invention.

In a further aspect, the invention relates to methods to facilitate the transfer of a polynucleotide or an anti-infective compounds into prokaryotic or eukaryotic organism for use in anti-infective therapy.

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

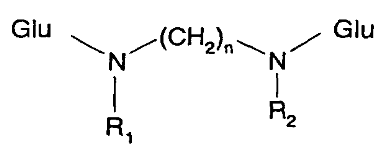
"Transfection" refers to the introduction of polynucleotides into cells in culture using methods involving the modification of the cell membrane either by chemical or physical means. Such methods are described in, for example, Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, N.Y. (1989). The polynucleotides may be linear or circular, single-stranded or double-stranded and may include elements controlling replication of the polynucleotide or expression of homologous or heterologous genes which may comprise part of the polynucleotide.

The invention will now be described by way of the following example.

Example 1 – Transfection of recombinant plasmid expressing luciferase into cells in culture using carbohydrate-based surfactant compounds.

Carbohydrate-based surfactant compounds having the general structure of formula (II)



(II)

were synthesised according to the method as hereinbefore described. The following compounds were made:

Compound no.	R_1-n-R_2
GS_G_1:	16-6-16
GS_G_3:	12-6-12
GS_G_4:	14-6-14
GS_G_5:	14-4-14
GS_G_6:	16-4-16
GS_G_7:	12-4-12
GS_G_8:	18-4-18
GS_G_9:	18-6-18

Transfection studies were performed using the adherent cell line CHO-K1 (Puck et al. 1958). Complete medium consisted of MEM alpha medium supplemented with 10 % v/v foetal bovine serum and 1x L-Glutamine. All media and supplements were obtained from Life Technologies.

Stable transfected cell lines expressing β–galactosidase were generated by cotransfection of the plasmid pSV-β-Galactosidase Control Vector (Promega) with the

plasmid Selecta Vecta-Neo (R & D Systems) in a 10:1 ratio. Following G418 (Life Technologies) selection (0.8 mg ml^{-1}), candidate cell lines were tested for β–galactosidase activity (β–Gal Reporter Gene Assay, chemiluminescent; Roche Diagnostics).

***In Vitro* Gene Transfection.**

Cells were seeded into 96-well plates (Beckton Dickinson) 16-18 hours prior to transfection at an approximate density of 1 x 10^4 cells per well. For transfection, 64 ng of the luciferase reporter gene plasmid, pGL3-Control Vector (Promega) per well, was incubated with various concentrations of the carbohydrate-based gemini compounds. After 30 minutes incubation at RT, OPTI-MEM® medium (Life Technologies) was added to the transfection mixture and the solution placed on the cells (final volume per well: 100 µl). Following a 3 hour or over night incubation at 37°C, the transfection solution was replaced with complete medium and the cells incubated further at 37°C. Reporter gene assays were performed according to the manufacturer's guidelines (Roche Diagnostics) approximately 48 hours post transfection. Luminescence was measured in a Packard TopCount NXT Microplate Scintillation and Luminescence Counter. For normalization purpose, β–galactosidase activity (β–Gal Reporter Gene Assay, chemiluminescent; Roche Diagnostics) was measured and luciferase activity per β–galactosidase activity was calculated. The results are shown in Figures 1 and 2.

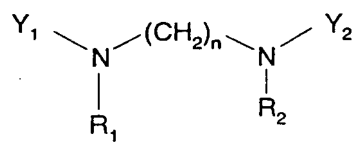
Brief description of the drawings.

Fig 1. Transfection of CHO-K1 cells (stable transfected with beta-galactosidase) with carbohydrate-based gemini compounds GS-G-3, GS-G-4, GS-G-5, GS-G-6, GS-G-7, GS-G-8, and GS-G-9. Concentrations of the compounds in μM is shown on the x axis. Bars represent the mean cps (counts per second) of 8 experiments ± the standard error of the mean.

Fig 2. Transfection of CHO-K1 cells (stable transfected with beta-galactosidase) with carbohydrate-based gemini compound GS-G-1. Concentrations of the compound in μM is shown on the x axis. Bars represent the mean cps (counts per second) of 8 experiments ± the standard error of the mean.

CLAIMS

1. The use of carbohydrate-based surfactant compounds having the general formula (I):



(I)

wherein Y_1 and Y_2, which may be the same or different, are carbohydrate groups; R_1 and R_2, which may be the same or different, are selected from:

a) hydrogen;

b) $C_{(1-24)}$ alkyl group; or

c) $C(O)-C_{(1-24)}$ alkyl carboxy group;

and n is from 1 to 10;

or a salt, preferably a pharmaceutically acceptable salt thereof,

for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into a eukaryotic or prokaryotic cell *in vivo* or *in vitro.*

2. The use according to claim 1 wherein the carbohydrate groups Y_1 and Y_2 are sugars.

3. The use according to claim 1 or 2 wherein R_1 and R_2 are alkyl groups of chain-length $C_{(10-20)}$ and n is between 2 and 8.

4. The use according to claim 3 wherein R_1 and R_2 are alkyl groups of chain-length $C_{(12-18)}$ and n is 4 or 6.

5. The use according to any one of claims 1 to 4 wherein the carbohydrate-based surfactant compound is symmetrical, that is the groups R_1 and R_2 are the same, and Y_1 and Y_2 are the same.

6. The use according to any one of claims 1 to 5 wherein the oligonucleotides or polynucleotides are transferred into cells to achieve an antisense knock-out effect.

7. The use according to any one of claims 1 to 5 wherein the oligonucleotides or polynucleotides are transferred into cells for gene therapy.

8. The use according to any one of claims 1 to 5 wherein the oligonucleotides or polynucleotides are transferred into cells for genetic immunisation (for the generation of antibodies) in whole organisms.

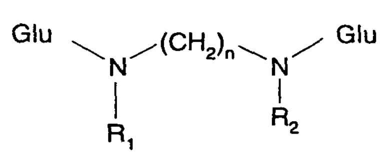
9. The use according to any one of claims 1 to 5 wherein the oligonucleotides or polynucleotides are transferred into cells in culture.

10. A carbohydrate-based surfactant compound as defined in claim 1 wherein R_1 and R_2 are alkyl groups of chain-length $C_{(10-20)}$ and n is between 2 and 8.

11. A carbohydrate-based surfactant compound according to claim 10 wherein R_1 and R_2 are alkyl groups of chain-length $C_{(12-18)}$ and n is 4 or 6.

12. A carbohydrate-based surfactant compound according to claim 10 or 11 wherein the carbohydrate-based surfactant compound is a gemini compound where R_1 and R_2 are the same and Y_1 and Y_2 are the same.

13. A carbohydrate-based surfactant compound according to claim 12 which has the formula (II):



(II)

wherein Glu is glucose in open chain form (glucitol).

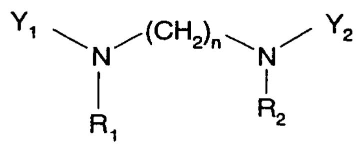
14. A carbohydrate-based surfactant compound of formula (I) in which one of R_1 or R_2 is an alkyl group of chain-length $C_{(1-24)}$, and the other is a $C_{(1-24)}$ alkyl carboxy group.

15. The use of a carbohydrate-based surfactant compound as defined in any one of claims 1 to 5 to facilitate the transfer of a polynucleotide or an anti-infective compound into a prokaryotic or eukaryotic organism for use in anti-infective therapy.

16. A process for preparing the carbohydrate-based surfactant compound of claim 10 comprising the addition of carbohydrate groups at the amine ends of an alkyl diamine compound.

Abstract

The use of carbohydrate-based surfactant compounds having the general formula (I):



(I)

wherein Y_1 and Y_2, which may be the same or different, are carbohydrate groups;

R_1 and R_2, which may be the same or different, are selected from:

a) hydrogen;

b) $C_{(1\text{-}24)}$ alkyl group; or

c) $C_{(1\text{-}24)}$ alkyl carboxy group;

and n is from 1 to 10;

for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*.

New carbohydrate-based surfactant compounds are also disclosed.

Figure 1

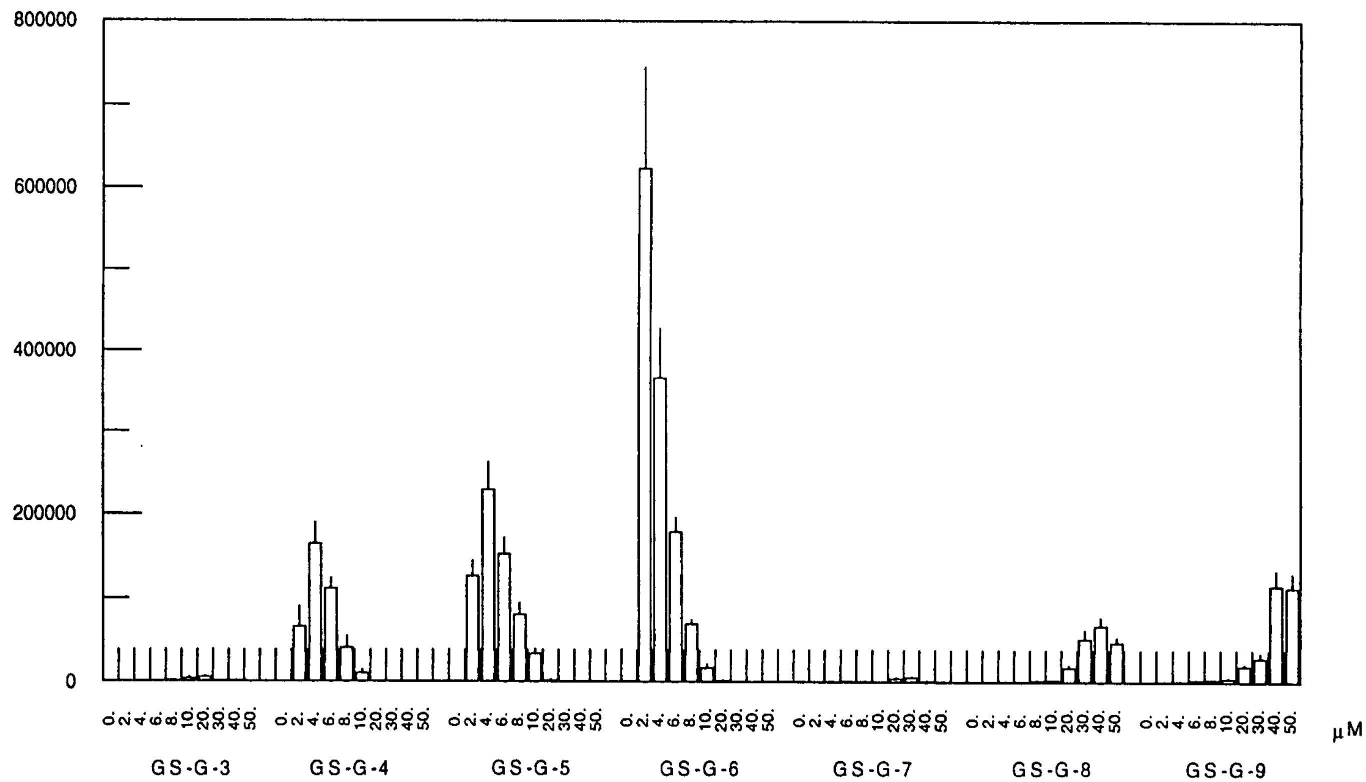
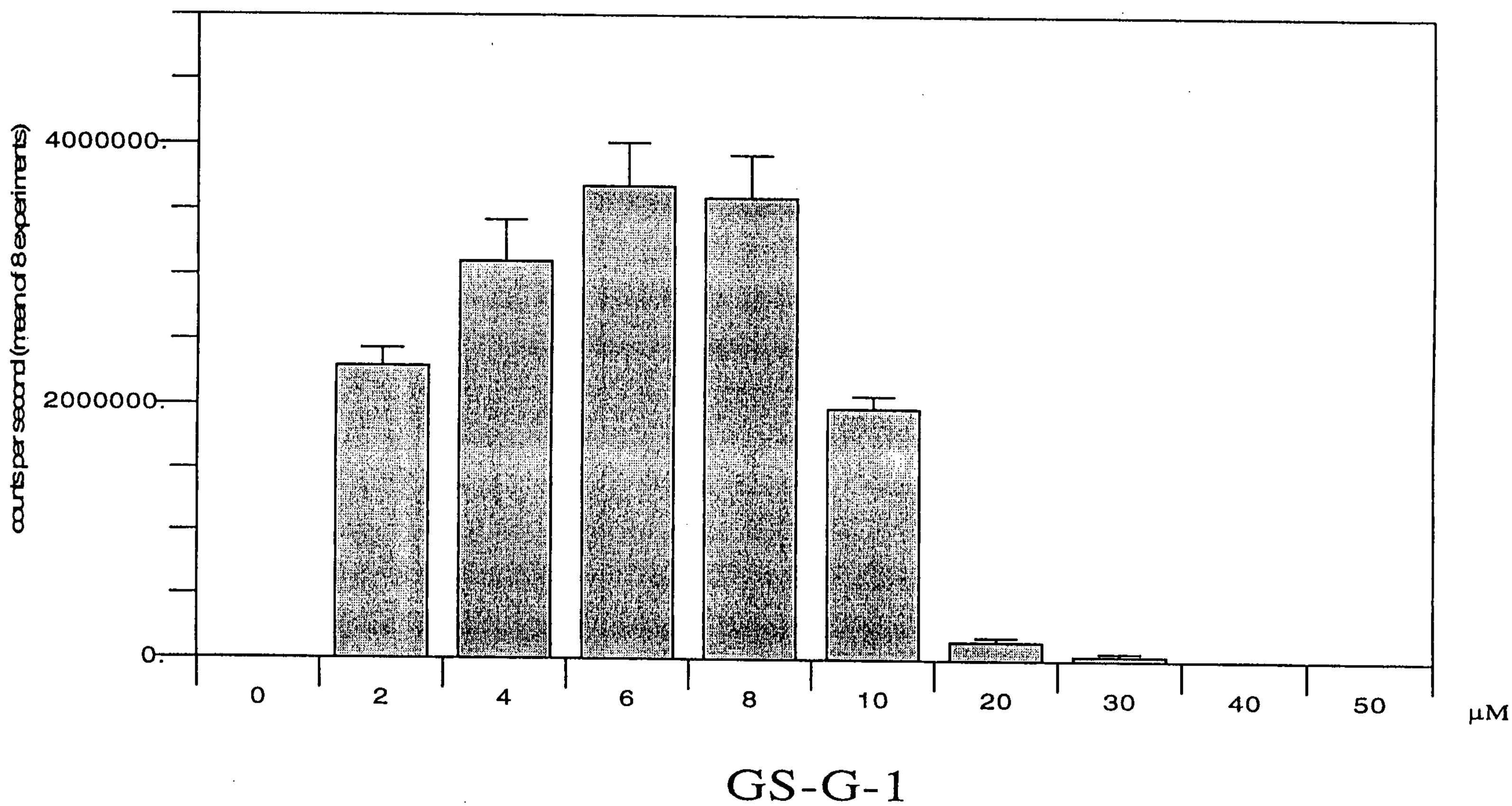

800000
600000
400000
200000
0
0. 2. 4. 6. 8. 10. 20. 30. 40. 50.
μM
GS-G-3
GS-G-4
GS-G-5
GS-G-6
GS-G-7
GS-G-8
GS-G-9

Figure 2


counts per second (mean of 8 experiments)
4000000
2000000
0
0
2
4
6
8
10
20
30
40
50
μM
GS-G-1

20 JUN 2000